FINAL REPORT

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Title: Radioimmunotargeting with Modified Streptavidin-Biotin

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For the past three years, we have designed and produced several useful streptavidin variants, characterized their properties both in vitro and in vivo, and developed methods and strategies needed for characterization of each molecule and the entire radioimmunotargeting approaches using these molecules. In molecular design of these streptavidin mutants, an enhanced molecular modeling method, including empirical free energy calculations (Vajda et al., 1994), was used which is of great assistance in designing streptavidin mutants effectively and intelligently. When the original proposal was submitted in 1992, the reviewers were rather skeptical about our proposal to design successfully a various streptavidin mutants, particularly a dimeric streptavidin and multi-flavor streptavidins. These were clearly very challenging tasks, but now we have the molecules in hand. This demonstrates the power of the molecular modeling method we are using and suggests the feasibility of further enhancements of these and other streptavidin variants by our molecular modeling capability. We have also developed signal amplification methods which could introduce large amounts of radioisotopes specifically at target tumor sites in the radioimmunotargeting approaches. These progresses are summarized below.

1. Molecular modeling and free energy calculations

Principles of free energy evaluation. The major obstacle in molecular design involving multi-subunit proteins or multi-element complexes is the need for binding free energy calculations which provide sufficient accuracy in predicting the effects of mutations and are computationally efficient for testing a substantial number of mutants. During the last three years, we have developed an enhanced empirical binding free energy evaluation model (Vajda et al., 1994). The binding free energy, ΔG , is decomposed according to: $\Delta G = E_{el} + \Delta G_d - T\Delta S_{sc} + \Delta G_{const}$, where E_{el} is the electrostatic interaction energy between two molecules, ΔG_d is the desolvation contribution to the binding free energy, and $T\Delta S_{sc}$ denotes the entropic term associated with the conformational entropy loss of the side chains of the reactants upon association. The last term, ΔG_{const} , includes rotational, translational, and cratic free energies and, as a first-order approximation, it was assumed to be constant at 11 kcal/mol.

The electrostatic interaction energy E_{el} is calculated by the Coulombic expression using a molecular mechanics potential function CHARMM (Brooks et al., 1988) with the distance-dependent dielectric permittivity $\epsilon = 4r$. The desolvation term ΔG_d is estimated by the atomic solvation parameter (ASP) model of Eisenberg and McLachlan (1986) as a linear combination of the accessible areas of different atoms using an updated set of solvation parameters (Vajda et al., 1995). The desolvation effect usually includes large negative hydrophobic contributions associated with the removal of non-polar atoms from water and smaller positive terms due to the desolvation of polar and charged atoms that become buried upon association of the reactants. The loss of side chain conformational entropy, ΔS_{sc} , is estimated by using an empirical entropy scale (Vajda et al., 1994).

The free energy model assumes good packing of both protein-protein and protein-solvent interfaces as seen in the X-ray structures of protein-protein complexes. Therefore, prior to free energy evaluation, the steric clashes are removed by subjecting the complex to energy minimization using the CHARMM potential. The binding free energy is calculated for natural tetrameric streptavidin using the known three-dimensional structure (Weber et al., 1989; Hendrickson et al., 1989) and various streptavidin variants whose models have been built using the geometric and force field parameters of the QUANTA/CHARMM program (Molecular Simulations, Inc.). We

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have extended the method to flexible ligands by taking into account the internal energy difference of the ligand between the bound and free states as well as the backbone entropy loss (Vajda, 1993).

Applications to protein-protein association. The direct evaluation of the method consisted of comparisons of calculated binding free energies with experimentally determined binding free energies. The simplest problem is calculating the binding free energies of complexes with known high-resolution X-ray structures, to which a rigid lock-and-key association model can apply. Proteases interacting with their inhibitors fall into this category. Because this comparison does not require structural prediction, it allows us to separate inadequacies in the free energy function from inadequacies in the search algorithms. The average difference between calculated and measured binding free energies is found to be approximately 1.3 kcal/mol, representing around 10% errors. This is somewhat smaller if the quantity of interest is the relative free energy of two close homologs, and hence the method is definitely adequate for the analysis of subunit interactions in a tetrameric streptavidin, and for the design of streptavidin mutants in which these interactions are altered.

The free energy function has also been used to predict free energy changes for molecules for which the structure of complexes are unknown (Weng et al., 1996). For example, we studied three protease-inhibitor pairs whose docked conformations were generated by Shoichet and Kuntz (1991) using either the complexed conformers (bound conformer docking) or the structures of isolated subunits or their close homologs (free conformer docking). The rigid body docking procedure successfully limits the number of potentially acceptable complex conformations to fewer than ten. However, members of the final set show substantial structural variability. Although they can differ as much as 10 Å, they are indistinguishable by traditional selection criteria. The free energy function was able to split the degeneracy and effectively identified the complexes that are close to the X-ray structure (Kuntz, 1992). Furthermore, the calculated free energies of the crystal structures are within 5% of the experimentally determined free energies.

Applications to protein-ligand interactions. Binding of a small ligand to its receptor protein often involves substantial conformational changes in the ligand, the receptor protein, or both. Hence, calculating the binding free energy is not as straightforward as for the protein-protein interactions (Rosenfeld et al., 1995; King et al., 1996). We attained acceptable accuracy in calculating binding free energies for twelve peptides bound to MHC class I receptors, but it was necessary to calculate the mean internal energy and desolvation free energy of the free peptides, averaging over the ensemble of free peptide conformations (Vajda et al., 1994). The problem is simpler if the goal is calculating the free energy change due to mutations or chemical modifications that do not substantially alter the structure of the complex. In particular, we determined relative binding free energies for the binding of two biotin analogs to streptavidin. The biotin analogs considered are 2-iminobiotin and thiobiotin (Vajda et al., 1994). The calculated relative free energies DDGh are about as accurate as the values recently obtained by free energy perturbation methods (Miyamoto and Kollman, 1993). However, the calculation requires only a few minutes rather than several days or even weeks of CPU time. Due to this computational efficiency, the method is useful for testing the effects of various mutations on binding. However, the rigid body model may not be applicable if the sizes of some side chains are altered or the structure of a biotin analog used differs substantially from that of biotin. Such changes can introduce cavities and/or flexible deformations that must be taken into account when calculating the binding free energy.

2. Design and production of core streptavidins

When streptavidin is produced by *S. avidinii*, it is secreted as a precursor, and then its signal peptide is cleaved off. The resulting mature protein is extremely susceptible to proteolysis, and thus both the N- and C-termini are readily truncated to yield so-called core streptavidin (Pähler et al., 1987; Bayer et al., 1989). However, natural core streptavidin preparations are often heterogeneous, and such structural heterogeneity can be observed even within single tetrameric

molecules. Thus, it would be desirable to make structurally homogeneous streptavidin for the application of this protein. In particular, we were interested in designing a minimum-sized core streptavidin which might have enhanced properties because of the removal of any non-functional terminal sequences that are located on the surface of the molecule.

Most natural core streptavidin molecules consist of the sequences from around Ala-13 to around Ser-139 (molecular mass, ~13.5 kDa per subunit), but the identity of the terminal residues varies. The sequences of natural core streptavidins are not necessarily those that are structurally most favorable; instead, they may merely indicate that the sequences around amino acids Ala-13 and Ser-139 are particularly susceptible to proteolysis. In fact, the X-ray crystallographic studies on natural core streptavidin were able to refine the structure only from Ala-13 or Glu-14 to Val-133 without the terminal regions (Weber et al., 1989; Hendrickson et al., 1989); this corresponds almost perfectly to the stable b-barrel structure consisting of the sequence from Gly-19 to Val-133. This indicates that the terminal regions, present in core streptavidin, are rather disordered (Weber et al., 1989), which suggests that they have little contribution to the fundamental properties of streptavidin, such as biotin binding, formation of the b-barrel structure, and subunit association. This prediction encouraged us to remove all of the terminal sequences, which have no apparent function, to design a minimum-sized core streptavidin. The recombinant core streptavidin we designed has the amino acid sequence from Gly-16 to Val-133 (molecular mass, 12.6 kDa) (Sano et al., 1995), and thus it consists of almost only the b-barrel structure characteristic of streptavidin.

This minimum-sized core streptavidin, termed Stv-13, is tetrameric and binds one biotin per subunit, as does natural streptavidin. The truncation of the terminal residues had no appreciable effects on the solubility characteristics of the protein. However, Stv-13 showed enhanced stability against denaturation by strong organic denaturants. For example, this molecule retained greater than 80% of its biotin-binding ability in the presence of 6 M guanidine hydrochloride at pH 1.5, under which conditions, natural core streptavidin lost almost all of its biotin-binding ability. In addition, Stv-13 had higher biotinylated DNA-binding ability than natural core streptavidin. These results suggest that the terminal regions, present on the surface of natural core streptavidin, reduce the overall stability of streptavidin and prevent biotinylated macromolecules from approaching the biotin-binding sites. Because of its enhanced properties, Stv-13 should be enormously useful for developing targeting reagents for this project. Thus, all of the streptavidin derivatives, produced in this project, are derived from this molecule.

The biodistribution of Stv-13 (50.4 kDa) was analyzed with the ^{111}In -labeled biotin analog, DB2 [diethylenetriaminepentaacetic acid a,w-bis(biocytinamide)] (Sigma) (Virzi et al., 1991), using normal CD-1 mice (Table 1). There is no significant difference in biodistribution between Stv-13 and natural core streptavidin (54 kDa) (Sigma). However, slight reduction in accumulation was seen in most organs, except the kidney. In addition, clearance of Stv-13 from the circulation was slightly faster than natural core streptavidin. These results suggest that the smaller size of Stv-13 is certainly useful for in vivo behavior, but a substantial further reduction in the size of streptavidin is needed to achieve favorable in vivo characteristics.

Table 1. Biodistribution of natural core streptavidin and the recombinant minimum-sized core streptavidin (Stv-13)

Organ	Natural core streptavidin	Stv-13
Liver	0.30(0.05)	0.29(0.06)
Heart	0.27(0.11)	0.18(0.06)
Kidney	4.5(3.1)	5.1(1.9)
Lung	0.44(0.31)	0.27(0.10)
Stomach	0.65(0.40)	0.30(0.12)
Spleen	0.20(0.08)	0.16(0.02)
Intestine	1.2(1.0)	0.98(0.54)
Muscle	0.29(0.29)	0.25(0.25)
Blood	0.87(0.33)	0.64(0.29)

N = 5. Biodistribution was analyzed at 5 hours after intravenous administration. Values are the percent injected doses per gram. Standard deviation of the mean is in parentheses.

3. Design and production of a streptavidin mutant with a reduced affinity for biotin

Trp-120 makes inter-subunit hydrophobic contacts to biotin bound by an adjacent subunit through the dimer-dimer interface. This inter-subunit contact is likely to make a key contribution to the extremely high biotin-binding affinity of streptavidin, because dissociated subunits have much lower affinity for biotin as seen by electrophoretic analysis of natural streptavidin (Sano et al., 1994) and with immobilized monomeric avidin known to release biotin under relatively mild conditions (Green and Toms, 1973). Interaction energy calculations also show that this residue makes relatively strong van der Waals interactions with biotin. To understand the role of the inter-subunit contacts made by Trp-120 to biotin in the properties of streptavidin, we designed a streptavidin mutant, in which Trp-120 is replaced with Phe-120 (Sano and Cantor, 1995). Our particular interest was to estimate the biotin-binding affinity of a dimeric streptavidin. Because a dimeric streptavidin lacks the inter-subunit contacts made by Trp-120 to biotin, the biotin-binding affinity of this mutant should be very close to that of a dimeric molecule.

This W120F streptavidin mutant, termed Stv-38, can still bind biotin tightly enough to fully retain it even in the presence of high concentrations of urea at neutral pH or in a wide pH range. However, its biotin-binding ability was reduced to the point where bound and free biotins show facile exchange under physiological conditions. The biotin-binding affinity (K_a) of this mutant was estimated to be $1 - 3 \times 10^8 \text{ M}^{-1}$, indicating the significant contribution of the inter-subunit contacts made by Trp-120 to biotin binding.

Biotin binding by natural streptavidin strengthens the dimer-dimer association by approximately 5 kcal/mol. Electrophoretic analysis of Stv-38 in the presence of sodium dodecyl sulfate indicates that its subunit association is not tightened significantly upon biotin binding, unlike natural streptavidin (Sano et al., 1994). The reduced effect of biotin binding on the subunit association of this mutant is apparently caused by the lack of the inter-subunit contacts made by Trp-120 to biotin through the weak dimer-dimer interface.

4. Design and production of cross-linked streptavidins

Natural tetrameric streptavidin has two subunit interfaces, the strong interface between subunits (monomer-monomer interface) in a stable dimer, and the weak dimer-dimer interface. If the subunit association, particularly at the weak dimer-dimer interface, could be tightened, the resulting streptavidin might retain bound biotin more stably even under relatively harsh conditions, because the maintenance of the tetrameric structure, particularly the dimer-dimer interface, is

essential for the inter-subunit contacts made by Trp-120 and Lys-121 to biotin. Such enhanced stability should be very useful for in vivo applications of streptavidin. To enhance the subunit association of streptavidin, specific covalent cross-links were introduced into pairs of subunits through the dimer-dimer interface (Reznik et al., 1996).

In natural tetrameric streptavidin, His-127 residues from two adjacent subunits are in close proximity across the weak dimer-dimer interface (Weber et al., 1989; Hendrickson et al., 1989). Replacing His-127 with Cys-127 generated a fully active, tetrameric protein, termed Stv-H127C, which can spontaneously form two disulfide bonds through the weak dimer-dimer interface under mild oxidizing conditions. The two pairs of adjacent cysteine residues can also be cross-linked irreversibly by a sulfhydryl-specific bifunctional cross-linker. The successful cross-linking of Cys-127 residues through the dimer-dimer interface without disturbing the biotin-binding ability indicates that two Cys-127 residues are located in close proximity, suggesting that no significant changes in local structure occurred by the mutation of His-127 to Cys-127 or by cross-linking between Cys-127 residues.

Another cross-linked mutant was made by first assembling a hetero-tetramer consisting of two species of subunits at a 1:1 ratio; one has lysine at position 127 (Stv-H127K) and the other has aspartic acid (Stv-H127D). These tetramers apparently formed predominantly with Lys-127 and Asp-127 apposed across the weak dimer-dimer interface; this configuration should be electrostatically more favorable than those having the same amino acid residues facing each other at the interface. This proposal has been confirmed by successful, almost quantitative cross-linking of the amino group of Lys-127 and the carboxyl group of Asp-127 by a zero-length cross-linker, a water-soluble carbodiimide. The resulting cross-linked molecule was essentially fully active.

These cross-linked streptavidins showed enhanced resistance to various denaturing conditions. For example, these streptavidins remained mostly tetrameric after heat treatment at 80 °C in the absence of biotin, under which conditions, natural core streptavidin dissociated completely into subunits. The cross-linked molecules retained bound biotin more stably than their counterparts without cross-links (or natural core streptavidin) in the presence of high concentrations of guanidine hydrochloride at very acidic pH. These results demonstrate that the introduction of covalent bonds between adjacent subunits through the weak dimer-dimer interface enhances the overall stability of streptavidin. They also reveal the importance of the inter-subunit contacts made by Trp-120 and Lys-121 to biotin through the dimer-dimer interface in biotin binding by streptavidin, which is consistent with the results seen with Stv-38 that has the mutation of Trp-120 to Phe-120 to reduce the inter-subunit contacts made by Trp-120 to biotin (Sano and Cantor, 1995). The biodistribution of these cross-linked streptavidins in normal CD-1 mice showed no significant difference from that of their parental molecule, Stv-13, as expected from that fact that all of these streptavidins have very similar molecular masses.

These studies on the reduced affinity streptavidin and cross-linked streptavidins not only generated useful streptavidin variants, but they have also shown our ability to design and modify the subunit interfaces of streptavidin to enhance its properties.

5. Design and production of a dimeric streptavidin

One of the major goals during this grant period is to design a streptavidin variant, which is much smaller than natural tetrameric streptavidin (~ 54 kDa). Natural streptavidin shows undesirable in vivo behavior, such as slow clearance from the circulation and non-specific accumulation in organs like the kidney and liver. This unfavorable in vivo behavior and pharmacokinetics must be caused primarily by the size of the molecule. Our strategy to overcome this problem is to design a dimeric streptavidin, which should be a more effective targeting reagent with faster clearance from the circulation and reduced non-specific accumulation.

To construct a dimeric streptavidin, we first replaced His-127 with a negatively charged amino, Asp. As described above, His-127 residues from adjacent subunits are in close proximity at the dimer-dimer interface. Replacing this residue with Asp-127 (termed Stv-33) should introduce strong repulsive electrostatic interactions into the dimer-dimer interface and prevent the formation of a tetrameric molecule. However, this single amino acid mutation did not produce a functional dimeric molecule. Free energy calculations (Vajda et al., 1994, 1995; Weng et al., 1996) based on the known three-dimensional structure of natural tetrameric streptavidin (Weber et al., 1989; Hendrickson et al., 1989) indicate that the major contribution to the association of subunits at the dimer-dimer interface is hydrophobicity, whereas the electrostatic attraction is relatively weak at -5.8 kcal/mol (Table 2). The mutation of His-127 to Asp-127 should make the electrostatic interaction repulsive ($E_{el} = +14.6$ kcal/mol). However, the desolvation free energy ΔG_d should remain very low (-64.2 kcal/mol), almost the same as that in natural tetrameric streptavidin (-68.9 kcal/mol). This indicates that the dimer-dimer interface region of Stv-33 is very hydrophobic; it suggests that dimeric Stv-33 should have very limited solubility in aqueous media. Production of a functional dimeric streptavidin will require additional mutations to reduce the hydrophobicity of the dimer-dimer interface region. Thus, the major obstacle in making a functional dimeric streptavidin was not the dissociation of a tetramer into two dimers; instead, the key questions were how to provide each subunit with the ability to fold into a functional dimeric form and how to improve the solubility of the dimeric molecule without disturbing its structure.

Several design strategies can be followed for improving the solubility characteristics of Stv-33. The most conservative strategy is to replace the hydrophobic residues in the dimer-dimer interface region with polar or charged amino acids. The most hydrophobic side chain that is exposed to solvent in a dimeric molecule is Trp-120. In natural tetrameric streptavidin, Trp-120 makes inter-subunit contacts to biotin bound by an adjacent subunit through the dimer-dimer interface. In a dimeric streptavidin, the latter subunit is no longer present, and this tryptophan ring should be fully exposed to solvent. Replacing Trp-120 with a polar or charged residue, in addition to the mutation of His-127 to Asp-127, will not reduce hydrophobicity to the level needed for sufficient solubility in aqueous media. For example, the mutation of Trp-120 to Asp-120 should raise the ΔG_d to -46.4 kcal/mol, but this may not high enough to provide the resulting protein with sufficient solubility. In fact, a similar streptavidin mutant, in which His-127 and Trp-120 have been converted to Asp-127 and Glu-120, respectively, formed insoluble aggregates upon renaturation (Sano et al., 1997). Free energy calculations reveal that at least two more hydrophobic side chains, Leu-124 and Val-125, must be replaced, but even this would be able to provide only limited solubility.

Table 2. Interaction free energies between subunits in natural tetrameric streptavidin and its dimeric mutants

(A) Subunit-subunit interaction energy through the dimer-dimer interface (kcal/mol)

Free energy term	Natural streptavidin	Stv-33	Stv-43
Electrostatic (E_{el})	-5.8	14.6	8.4
Desolvation (ΔG_d)	-68.9	-64.2	-23.4
Entropic ($-T\Delta S_{sc}$)	27.9	27.1	5.6
Total	-46.8	-22.8	-9.4

(B) Subunit-subunit interaction energy through the monomer-monomer interface (kcal/mol)

Free energy term	Natural streptavidin	Stv-33	Stv-43
Electrostatic (Eel)	- 22.3	- 21.8	- 12.8
Desolvation (ΔG_d)	- 66.9	- 66.9	- 55.1
Entropic (- $T\Delta S_{sc}$)	50.4	50.4	42.3
Total	- 38.8	- 38.3	- 25.6

Here we used a different strategy, in which the entire loop region consisting of the sequence from Gly-113 through Trp-120 is removed, in addition to the mutation of His-127 to Asp-127 (Fig. 1) (Sano et al., 1997). In natural streptavidin, the carbonyl carbon of Ser-112 and the amide nitrogen of Lys-121 are only 9 Å apart, and this gap should be easily closed by adjusting the conformations of these two residues. Therefore, deletion of the sequence from Gly-113 to Trp-120 by fusing Ser-112 to Lys-121 should have little effect on the subunit structure. The resulting molecule should still maintain a positive ΔE_{el} for association between the two dimers via the dimer-dimer interface (8.4 kcal/mol; see Table 3), which should prevent the formation of a tetrameric molecule. The ΔG_d of the dimer-dimer interface region of this mutant (- 23.4 kcal/mol) is considerably higher than those of Stv-33 (- 64.2 kcal/mol) and natural tetrameric streptavidin (- 68.9 kcal/mol), suggesting that this molecule could maintain the dimeric structure with sufficient solubility in aqueous media.

Based on the molecular modeling described above, a streptavidin mutant, Stv-43, in which the loop from Gly-113 to Trp-120 has been deleted in addition to the mutation of His-127 to Asp-127, was designed. Stv-43 was expressed efficiently in *E. coli* by using the T7 expression system (Studier et al., 1990). Purification of expressed Stv-43 was carried out by the method used for other recombinant streptavidin variants. However, this method was unable to purify a functional dimeric streptavidin. Thus, modifications were made in the purification procedure. The major modification is to include excess biotin in all solutions during renaturation. The presence of biotin might allow denatured, monomeric Stv-43 to fold correctly around a biotin molecule which would act like a molecular chaperon. Biotin binding would also provide Stv-43 with additional solubility in aqueous media by sealing hydrophobic amino acid residues in the biotin-binding site and by providing an additional negative charge derived from the carboxyl group of biotin that is exposed to solvent.

The addition of biotin during renaturation allowed successful renaturation of Stv-43. After theremoval of bound biotin for which a very low concentration of urea was used as a biotin analog known to have a low affinity for avidin (Green, 1970), Stv-43 in the form of a biotin complex was purified to homogeneity by biotin affinity chromatography. Purified Stv-43 showed a single band at about 13 kDa on SDS-PAGE and a single peak at 23 kDa on gel filtration chromatography using a Superdex 75 HR 10/30 column (Pharmacia). Purified Stv-43 in the presence of excess biotin was stable at 4 °C. However, in the absence of bound biotin, Stv-43 gradually dissociated into monomeric forms after several days of storage at 4 °C. Gel filtration chromatography of Stv-43, which had been stored without bound biotin, shows that only the remaining dimeric Stv-43 bound biotin, while no biotin binding was detected with the monomeric forms. This suggests that the monomeric molecules, dissociated from the active dimeric protein, cannot maintain the functional three-dimensional structure. Free energy calculations on the subunit interaction at the monomer-monomer interface of Stv-43 (Table 2) show that, in the absence of biotin, its ΔE_{el} should be significantly higher (- 12.8 kcal/mol) than that of the equivalent subunit pair in tetrameric streptavidin (- 22.3 kcal/mol). This suggests that the monomer-monomer interaction of Stv-43 may be weakened by the mutations made, and that it has insufficient stability to maintain its dimeric

structure without bound biotin. Once the dissociation of subunits occurs, the resulting monomeric molecule is likely to denature because the b-barrel structure, characteristic of streptavidin subunits, would not be maintained stably without the interaction with the adjacent subunit which has a complementary curvature at the monomer-monomer interface and makes numerous inter-subunit van der Waals interactions.

Purified Stv-43 without biotin bound greater than 0.95 molecules of biotin per subunit, indicating that this protein has full biotin-binding ability. However, the biotin-binding affinity constant (K_a) of Stv-43 was reduced to $1.5 \times 10^7 \text{ M}^{-1}$ at 4°C at pH 7.4. This is approximately one order of magnitude smaller than that of a tetrameric streptavidin mutant, Stv-38, in which the mutation of Trp-120 to Phe-120 is made to weaken the inter-subunit contacts made by Trp-120 to biotin (Sano and Cantor, 1995). This suggests that Phe-120 in Stv-38 makes weak inter-subunit contacts to biotin bound by an adjacent subunit through the dimer-dimer interface. The biotin-binding affinity constant of Stv-43 is in good agreement with that of a tetrameric streptavidin mutant ($8.6 \times 10^6 \text{ M}^{-1}$) (Chilkoti et al., 1995), in which Trp-120 is converted to Ala-120 and thus inter-subunit contacts made by Trp-120 to biotin through the dimer-dimer interface are disrupted almost completely (note, however, that the conditions used for the determination of biotin-binding affinity by Chilkoti et al. are different from our conditions). These comparisons reveal that inter-subunit contacts made by Trp-120 and Lys-121 to biotin bound by an adjacent subunit through the dimer-dimer interface make a considerable contribution to the extremely tight biotin binding by tetrameric streptavidin. They also demonstrate the importance of subunit association and inter-subunit communications in the biotin-binding characteristics of streptavidin.

The biodistribution of this dimeric streptavidin was tested by using normal CD-1 mice. Stv-43 was labeled with ^{111}In via a biotin derivative, DB2 (Sigma). The biodistribution of the ^{111}In -labeled Stv-43 was analyzed at 3 hours after administration, and the result is summarized in Table 3. As expected, this dimeric streptavidin has much faster clearance from the circulation, and non-specific accumulation in the organs analyzed is significantly reduced. Particularly, the accumulation of Stv-43 in the kidney and liver is considerably reduced to 2.78% and 0.09%, respectively, of the injected dose per gram tissue, as compared with that of the minimum-sized tetrameric core streptavidin (kidney, 5.1%; liver, 0.29%) (see Table 3). These results demonstrate that this dimeric streptavidin has much enhanced in vivo properties, suggesting its great potential to become an enhanced targeting reagent for radioimmunotargeting approaches.

Table 3. Biodistribution of a dimeric streptavidin, Stv-43, along with the minimum-sized tetrameric core streptavidin (Stv-13)

Organ	Stv-43	Stv-13
Liver	0.09(0.01)	0.29(0.06)
Heart	0.02(0.00)	0.18(0.06)
Kidney	2.78(1.66)	5.1(1.9)
Lung	0.14(0.09)	0.27(0.10)
Stomach	0.06(0.04)	0.30(0.12)
Spleen	0.02(0.01)	0.16(0.02)
Intestine	0.01(0.01)	0.98(0.54)
Muscle	0.06(0.03)	0.25(0.25)
Blood	0.08(0.09)	0.64(0.29)

N = 5. Biodistribution was analyzed at 3 hours and 5 hours after intravenous administration for ^{111}In -labeled Stv-43 and Stv-13, respectively. Values are the percent injected doses per gram. Standard deviation of the mean is in parentheses.

To improve the properties of Stv-43, particularly the greater folding and binding ability, additional molecular modeling has been made. New dimeric streptavidins, based on the molecular modeling, are being constructed. These are described in the Research Design and Methods section below.

6. Design and production of multi-flavor streptavidins

When streptavidin or its derivatives are used as targeting reagents for biotinylated molecules in vivo, the most serious problem is endogenous biotin. Because of its extremely high-affinity biotin binding, streptavidin binds endogenous biotin spontaneously after administration to the body, and it does not release bound biotin under physiological conditions. This means that no biotin-binding sites are available for binding to biotinylated targets. To overcome this problem, we have designed and produced streptavidin derivatives that bind biotin analogs much tighter than natural biotin.

We have designed streptavidin mutants for two commercially available biotin analogs, 2-iminobiotin and diaminobiotin (cis-3,4-diamino-2-tetrahydrothiophanyvaleric acid) by the free energy evaluation method (Vajda et al., 1994). Since these biotin analogs retain substantial structural features of biotin, the known three-dimensional structure of the streptavidin-biotin complex (Weber et al., 1989; Hendrickson et al., 1989) was used as a template. The design of streptavidin mutants involved amino acid substitutions at specific amino acid residues responsible for biotin binding. The search for the most probable side chain positions of the mutated amino acid residues was carried out using molecular modeling package QUANTA (Molecular Simulations, Inc). The resulting structures were refined by minimizing the conformational energy of the complex using the CHARMM force field (Brooks et al., 1988). The binding free energy for each mutant has been calculated as described.

In 2-iminobiotin, the ureido oxygen ($q = -0.65$ eV) of biotin is replaced with an amino group (guanidino group) that is protonated at pH 7. This results in a strong dipole of 1.4-eV charge on the guanidino hydrogens and a charge of -1.04 eV on the guanidine nitrogen. In diaminobiotin, the ureido group is removed, and the two amino groups are somewhat positively charged. Figure 1 shows biotin, and the amino acid residues of natural streptavidin that have direct contacts to biotin. The primary design strategy is based on the idea that the substitution of Ser-27 by Asp-27 should yield unfavorable electrostatic interactions with biotin, while improving the binding of 2-iminobiotin and diaminobiotin. If Ser-27 is replaced with Glu-27, additional steric interactions will further reduce biotin binding. This mutation should have only slight effects on the binding of 2-iminobiotin and have almost no effect on the binding of diaminobiotin. The affinity of streptavidin mutants for 2-iminobiotin and diaminobiotin should be further increased by introducing additional mutations into Ser-45 and Asn-23.

Based on the molecular design described above, two streptavidin mutants have been constructed. One streptavidin mutant has the mutations of Ser-27 and Asn-23 to Asp-27 and Ala-23, respectively (Stv-A23D27). The other mutant has the mutation of Ser-27 to Glu-27, in addition to the substitution of Asn-23 to Ala-23 (Stv-A23E27). These two streptavidin mutants were expressed very efficiently in *E. coli* by using the T7 expression system (Studier et al., 1990). Purification of these streptavidin mutants was carried out by affinity chromatography using diaminobiotin as the ligand. Both mutants bind to diaminobiotin at pH 6.0. The release of Stv-A23D27 and Stv-A23E27 was done with a solution containing urea at 10.0 and a similar solution at pH 9.0, respectively. Stv-A23D27 was released very efficiently under these conditions, while the yield of Stv-A23E27 was low for unknown reasons. Modifications are currently being made in the purification procedure for Stv-A23E27 to improve the yield.

Further characterization of Stv-A23D27 has been carried out. Stv-A23D27 binds 2-iminobiotin in the pH range from 4 to 13 with the maximum binding at pH between 8 and 10. This streptavidin mutant binds diaminobiotin in a narrower pH range from 4 to 9. When this mutant is bound to 2-iminobiotin, the addition of diaminobiotin does not cause significant exchange of bound 2-iminobiotin with diaminobiotin. Similarly, bound diaminobiotin showed no significant exchange with 2-iminobiotin. These indicate that Stv-A23D27 binds both 2-iminobiotin and diaminobiotin tightly, and its affinities for these biotin analogs may be at similar levels. The affinity (Kd) of Stv-A23D27 for natural biotin, analyzed by using ¹⁴C-labeled biotin, is in a range of 10⁻³ - 10⁻⁴ M, indicating that this mutant has only very limited binding affinity for biotin.

To determine the binding affinity of this mutant for 2-iminobiotin, we prepared radiolabeled 2-iminobiotin (note that radiolabeled 2-iminobiotin and diaminobiotin are not commercially available). Cystamine [S2(CH₂CH₂NH₂)₂] (Sigma) was attached to agarose beads containing activated N-hydroxysuccinimidyl groups (NHS-agarose) (Pharmacia) via one of the amino groups. A 2-iminobiotin analog containing an NHS group at the carboxyl end (NHS-iminobiotin) (Sigma) was attached to the unreacted amino group of cystamine that had been immobilized on NHS agarose. After unbound materials were removed, the disulfide bond of cystamine was cleaved by dithiothreitol (DTT) and released from the agarose beads. Eluted half cystamine (= cysteamine; H₂NCH₂CH₂SH) conjugated to the carboxyl end of 2-iminobiotin via the amino group was labeled with ¹⁴C by S-carboxymethylation of the reactive SH group of the cysteamine moiety with iodo[2-¹⁴C]acetic acid. The resulting radiolabeled 2-iminobiotin analog was used to estimate the affinity of Stv-A23D27 for 2-iminobiotin. Preliminary data indicate that this streptavidin mutant has a binding affinity (Kd) for 2-iminobiotin of 10⁻⁷ - 10⁻⁸ M. This reveals that Stv-A23D27 binds 2-iminobiotin with great preference over natural biotin. These results suggest that the use of this streptavidin mutant or its derivatives should be able to overcome problems caused by endogenous biotin in radioimmunotargeting applications.

Because this radiolabeled 2-iminobiotin has relatively low specific radioactivity, the estimation of the binding affinity of this streptavidin mutant is inaccurate. In addition, once the affinity of these streptavidin mutants for these biotin analogs are further enhanced during this proposed project, the ¹⁴C-labeled 2-iminobiotin analog may not be usable. Thus, another 2-iminobiotin analog radiolabeled with ³⁵S, which should have at least 104 times higher specific radioactivity than the ¹⁴C-labeled analog, is being prepared by using a similar conjugation method. Similarly, an ³⁵S-labeled diaminobiotin analog is also prepared.

7. Labeling and biodistribution of a streptavidin-metallothionein chimera

Metallothionein is a small (~ 6 kDa), cysteine-rich protein that can bind a variety of heavy metal ions with high affinity (for reviews, see Hammer, 1986; Kägi and Schaffer, 1988). A fusion protein between streptavidin and metallothionein was prepared (Sano et al., 1992) to provide metallothionein with biological recognition specificity. This streptavidin-metallothionein chimera could be very useful as a radionuclide targeting reagent to tumor cells with surfaces labeled with a

biotinylated monoclonal antibody against a tumor-associated antigen. To test this, the chimera was labeled with ^{99m}Tc and ^{188}Re , which have desirable radiation characteristics for imaging and therapy of cancer, respectively, and the radiolabeled chimera was characterized both *in vitro* and *in vivo* (Virzi et al., 1995).

The streptavidin-metallothionein chimera was labeled by transchelation with ^{99m}Tc - and ^{188}Re -glucoheptonate, with labeling efficiencies of 60% and 20%, respectively. The radiolabeled chimeras retain their binding ability for a biotinylated monoclonal antibody, indicating that radiolabeling has no appreciable effect on the fundamental properties of streptavidin. When radiolabeled chimeras were incubated in fresh serum at 37°C for 24 hours, only about 20% of the ^{99m}Tc label was released from the chimera probably by transchelation to cysteine in the serum. In contrast, the ^{188}Re label showed lower stability in serum, and approximately 40% of the label was transchelated from the chimera to serum-derived cysteine or other SH-containing materials. The biodistribution of the radiolabeled chimeras was also analyzed by using normal CD-1 mice (Table 4). The biodistribution of the ^{99m}Tc -labeled chimera has no significant difference from that of the ^{188}Re -labeled chimera. However, because of the large size of this chimera (~ 80 kDa), clearance of the radiolabeled chimeras from the circulation was very slow, and significant non-specific accumulation in the liver, kidney, and spleen was seen.

These results reveal that the streptavidin-metallothionein chimera, when radiolabeled with appropriate metal radioisotopes, can be used as an effective radionuclide carrier in radioimmunotargeting applications, if a relatively short targeting time, such as less than one day, is used to avoid transchelation of the radiolabels. They also demonstrate the need for the production of smaller targeting molecules to achieve faster clearance from the circulation and to reduce non-specific accumulation in organs, such as the liver and kidney.

Table 4. Biodistribution of the streptavidin-metallothionein chimera labeled with ^{99m}Tc and ^{188}Re

Organ	^{99m}Tc	^{188}Re
Liver	6.0(0.9)	6.5(0.7)
Heart	2.8(0.5)	1.7(0.3)
Kidney	5.9(1.5)	6.7(1.1)
Lung	3.5(0.6)	2.3(0.2)
Stomach	0.9(0.2)	0.7(0.1)
Spleen	12(5.0)	4.8(0.7)
Muscle	0.5(0.2)	0.3(0.03)
Blood	13(3.0)	14(7.0)

N = 6 and 5 for the chimera labeled with ^{99m}Tc and ^{188}Re , respectively. Biodistribution was analyzed at 5 hours after intravenous administration. Values are the percent injected doses per gram. Standard deviation of the mean is in parentheses.

8. Effect of charges on the biodistribution of streptavidin

The *in vivo* behavior of proteins depends on many factors. As seen with the biodistribution of the minimum-sized core streptavidin Stv-13, the dimeric streptavidin Stv-43, and the streptavidin-metallothionein chimera, the size of the molecule is the primary factor. In addition to this, the surface charges of the molecule also affect its *in vivo* behavior. For example, negative charges on a molecule reduce its binding to other molecules in the body, because the majority of biological molecules are negatively charged. To test if the modification of surface charges of

streptavidin could alter the biodistribution, negative charges were introduced. A streptavidin mutant containing a stretch of five cysteine residues per subunit (twenty cysteines per molecule) (Sano et al., 1993) was alkylated by S-carboxymethylation with iodoacetic acid (Ac-Stv). This provides each streptavidin molecule with twenty additional negative charges. As a control, the same streptavidin mutant was alkylated with iodoacetamide (Am-Stv), which does not alter the charge. The biodistribution of these modified streptavidins was analyzed in normal mice by using ^{111}In -labeled biotin analog DB2 (Table 5).

Table 5. Biodistribution of alkylated streptavidins (Ac-Stv and Am-Stv) and minimum-sized core streptavidin (Stv-13)

Organ	Ac-Stv	Am-Stv	Stv-13
Liver	0.50(0.48)	3.9(1.2)	0.29(0.06)
Heart	1.7(3.5)	0.3(0.12)	0.18(0.06)
Kidney	3.6(3.1)	4.2(3.5)	5.1(1.9)
Lung	0.26(0.15)	0.23(0.07)	0.27(0.10)
Stomach	1.4(2.6)	0.25(0.13)	0.30(0.12)
Spleen	0.22(0.11)	0.96(0.34)	0.16(0.02)
Intestine	2.1(3.0)	0.40(0.25)	0.98(0.54)
Muscle	0.08(0.05)	0.09(0.04)	0.25(0.25)
Blood	0.35(0.14)	0.18(0.05)	0.64(0.29)

N = 5. Biodistribution was analyzed at 3 hours after intravenous administration. Values are the percent injected doses per gram. Standard deviation of the mean is in parentheses.

The modified streptavidins showed slightly different biodistribution patterns from the minimum-sized core streptavidin Stv-13. For example, Ac-Stv showed more accumulation in the liver, heart, stomach, and intestine than Stv-13, but it had significantly reduced accumulation in the kidney and muscle. In contrast, Am-Stv had significant higher accumulation in the liver and spleen, but it showed reduced accumulation in the kidney, intestine, and muscle. Both Ac-Stv and Am-Stv showed faster clearance from the circulation than Stv-13. Although these results do not lead to definitive conclusions about the effect of charges on the biodistribution of streptavidin, they imply that modifications of surface charges of streptavidin could be used to alter the *in vivo* behavior of streptavidin.

9. Labeling and biodistribution of DNA molecules

Several unique properties of DNA make it one of the most easily manipulated biological materials. Among these properties are: (i) DNA is one of the most monotonous biological molecules, because it consists of only four basic elements; (ii) Single-stranded DNA molecules recognize their complements with great specificity to form double-stranded DNA; (iii) DNA can be easily modified by both chemical and enzymatic means; (iv) Relatively short single-stranded DNA molecules, up to about one hundred bases long, can easily be generated chemically by using an automated DNA synthesizer; and (v) DNA can be amplified biologically or enzymatically. These unique properties suggest relatively short DNAs, i.e., oligonucleotides, could be very useful as radionuclide delivery carriers in radioimmunotargeting applications. For example, a radiolabel could be attached to a single-stranded oligonucleotide, which could be delivered specifically to tumor cells marked with its complementary sequence.

We have developed a method for labeling oligonucleotides with $^{99\text{m}}\text{Tc}$, an ideal radionuclide for cancer imaging (Hnatowich et al. 1995). This method uses an NHS (N-

hydroxysuccinimide) derivative of SHNH (hydrazinonicotineamide) as a metal chelator that is attached to a primary amino group at the terminus of an oligonucleotide. By using this radiolabeling method, a 22-base single-stranded oligonucleotide with the natural phosphodiester backbone and its derivative with phosphorothioate linkages were labeled with ^{99m}Tc (Hnatowich et al., 1996). When these radiolabeled oligonucleotides were incubated in serum, rapid binding of serum proteins was observed. The primary protein-binding site is likely to be the SHNH chelator, not the DNA moiety. In phosphorothioate DNA, the reactive sulfur atoms of the phosphorothioate groups may also bind to serum components (Sands et al., 1994; Ghose et al., 1993; Cossum et al., 1993). Serum protein binding apparently stabilizes the DNAs with high stability against nucleases.

These radiolabeled DNAs and the same sequences with mixed phosphodiester and phosphorothioate backbones (the three 3'-terminal phosphodiester linkages were changed to phosphorothioates) were administered intravenously into normal mice to test their *in vivo* behavior (Table 6). The phosphodiester and phosphodiester/phosphorothioate DNAs accumulated in the kidney immediately after the administration. In contrast, the phosphorothioate DNA showed much higher accumulation in the liver, kidney, and spleen.

Table 6. Biodistribution of ^{99m}Tc -labeled phosphodiester, phosphorothioate, and phosphodiester/phosphorothioate DNAs

Organ	Phosphodiester	Phosphorothioate	Phosphodiester/phosphorothioate
Liver	0.9(1.0)	47(12)	8.0(4.5)
Heart	0.12(0.04)	1.4(0.9)	0.9(0.5)
Kidney	0.5(1.2)	30(5.7)	21(12)
Lung	0.29(0.08)	2.3(0.8)	1.7(0.9)
Stomach	0.33(0.05)	2.4(0.8)	4.1(1.8)
Spleen	0.78(0.39)	22(5.0)	4.1(2.1)
Blood	0.24(0.02)	2.4(0.5)	3.6(2.5)

N = 4 for phosphodiester DNA and the phosphodiester/phosphorothioate DNA. N = 3 for phosphorothioate DNA. Biodistribution was analyzed at 4 hours after intravenous administration. Values are the percent injected doses per gram. Standard deviation of the mean is in parentheses.

10. Signal amplification and assembly systems using streptavidin and nucleic acids

We have initiated several different approaches to develop signal amplification and assembly systems for *in vitro* diagnostics and as novel materials for bioseparations, currently supported by other funding sources. This will pave the way for *in vivo* tests of these systems as part of the proposed continuation of this project. Three major molecular species are being tested. The first is streptavidin because of its extremely tight biotin-binding ability and the D2 symmetry of the molecule. The second is nucleic acids because of their unique properties described in the preceding section. The third is peptides. A group of peptides can recognize particular peptide sequences, and this recognition capability is used by nature to generate multi-subunit proteins.

Streptavidin-DNA conjugates as self-assembling systems. The streptavidin tetramer has the D2 symmetry (Pähler et al., 1987; Weber et al., 1989; Hendrickson et al., 1989). This structural characteristic, i.e., a pseudo-tetrahedral structure, could allow streptavidin to serve as a building block of a self-assembly system by combining it with other recognition elements. We have developed a method to attach covalently a short 5'-thiolated DNA to streptavidin using a hetero-bifunctional cross-linker, sulfo-SMPB [sulfosuccinimidyl 4-(p-maleimidophenyl)butyrate; Pierce]

which reacts with sulfhydryl and primary amino groups (Niemeyer et al., 1994). This hetero-bifunctional cross-linker allows the covalent attachment of the 5'-sulfhydryl group of the DNA to a primary amino group of streptavidin. Although the streptavidin conjugation sites have not yet determined, they are most likely the N-terminus because this is located on the surface of the molecule, while other primary amino groups, derived from lysine residues, have very limited accessibility. The resulting streptavidin-DNA conjugates can be fractionated by ion exchange chromatography, in which each conjugate species, containing one to four DNA molecules per streptavidin, can be isolated.

These streptavidin-DNA conjugates are very useful in many applications, which use both the very high specificity of streptavidin to biotin and that of single-stranded DNA to its complementary sequence. For example, self-assembling bispecific antibodies were prepared by simply mixing one streptavidin-DNA conjugate containing a biotinylated antibody with another streptavidin-DNA conjugate containing a different biotinylated antibody, where the sequences of the attached DNA molecules are complementary. Self-sorting protein chips, on which different proteins are immobilized in an ordered manner, were prepared by using streptavidin-DNA conjugates, each containing a different biotinylated protein and a unique DNA sequence that is complementary to one of immobilized capturing DNA sequences. Linear protein arrays were prepared by annealing several streptavidin-DNA conjugates, each containing a different biotinylated protein and a unique DNA sequence, along a long single-stranded template nucleic acid containing sequence segments complementary to those of the streptavidin-DNA conjugates.

Streptavidin-peptide chimeras for self-assembling systems. As described above, a group of peptides can specifically recognize particular peptide sequences. This recognition capability is often used by nature to generate multi-subunit proteins. A well-studied example is the leucine zipper seen in many dimeric transcription regulation proteins. We are using leucine zipper sequences (O'Shea et al., 1989, 1991, 1992; Zhou et al., 1992; Monera et al., 1993), each of which is fused to the N- and C-terminus of streptavidin. Each of the resulting chimeric proteins should have four identical recognition sequences that are located at the corner of a tetrahedron. A key consideration here is that each chimeric protein species alone has no assembly capability. However, once it is mixed with the other species, they recognize each other through the leucine zipper sequences and form aggregates. Another key issue here is that the biotin-binding sites of the streptavidin moiety are fully open. Thus, any biotinylated molecules can be targeted to these molecules after aggregates have been formed.

We have already constructed an N-terminal fusion of streptavidin with one of the leucine zipper strands, and we are currently making a C-terminal fusion. Once the latter has been constructed, these two chimeric proteins will be tested to see if they can form aggregates upon mixing, and we will refine the system to be able to control the dimension of aggregates and the uniformity and homogeneity of aggregation processes.

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A.

B.

Figure 1. A. Streptavidin tetramer. One of the subunits is shown as a solid line. Side chains are indicated only for segment 106 - 128, which is of interest in the design of dimeric streptavidins. B. Truncated loop region of Stv-43, i.e., after deleting residues 113 - 121 (solid line), superimposed on the segment 106 - 128 of the original streptavidin (dashed line). Residue His-127 from the adjacent subunit is also shown (thin line).

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